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14. ABSTRACT

Our laboratory has been interested in the role of Akt in the development of hormone-independent cancers. Using a breast cancer cell model, we previously demonstrated that tumors with a constitutively active Akt are resistant to anti-hormone therapy. In this study we have expanded upon our preliminary observations in the breast model into in vitroprostate cancer models to determine the molecular and biological mechanisms underlying these findings. In our second year of this study, we found that treatment with an Akt inhibitor prevented the progression of LNCaP cells to a state of androgen-independence. These results correlated with suppression of expression of the androgen receptor, as well as suppression of the pro-survival proteins bcl-2 and NF-kB. We are currently exploring the significance of these findings in relationship to the preventive properties of the omega-3 fatty acids. Currently, progression of prostate cancer to androgen independence remains the primary obstacle to improved survival with this disease. The results of our studies suggest that targeting the Akt pathway may provide a strategy for preventing progression, resulting in increased survival among patients with recurrent disease.

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INTRODUCTION

Our laboratory has been interested in the role of Akt in the development of hormone-independent cancers. Using a breast cancer cell model, we have demonstrated that tumors with a constitutively active Akt are resistant to anti-hormone therapy. In this study we will expand our preliminary observations in the breast model into *in vitro* and *in vivo* prostate cancer models and determine the molecular and biological mechanisms underlying these findings.

BODY:

Task 1*: To determine whether the level of phospho-Akt within the tumor is a predictor of eventual development of hormone-refractory disease.*

Perform immunohistochemical staining and analyses of paraffin-imbedded core prostate biopsies from two cohorts of patients: 1) those that did develop hormone refractory metastatic disease and 2) those that did not develop hormone refractory metastatic disease

We have now compiled the biopsy samples that will be evaluated for this Specific Aim. We are currently conducting the immunohistochemical studies.

Task 2: *To investigate in vitro whether Akt signaling is a critical component of one of the mechanisms by which prostate cancer progresses to a condition of hormone independence.*

Culture LNCaP and CRW-R1 cells under conditions of hormone ablation with and without co-treatment with an Akt inhibitor and assess for progression as well as alterations in cell cycle, apoptosis and signal transduction.

As was reported last year, we have completed this task. The results of this component of the study have been reported at the annual meeting of the American Institute for Cancer Research, the annual meeting of the American Association for Cancer Research, and the EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics". Two manuscripts are in preparation for a April submission to Clinical Cancer Research and Cancer Research.

In the previous progress report, we presented the results of our hormone ablation studies, in which we found that treatment with the Akt inhibitor prevented the progression of LNCaP cells to a state of androgen-independence. As seen in **Fig. 1**, all but one of the clones exposed to the Akt inhibitor arrested by week 5, and never recovered. Conversely, only seventeen (43%) of the clones in the charcoal-stripped alone group (CSS) continuously arrested. Fourteen (40%) of the clones in the CSS group arrested, but then recovered, suggesting that this subset is now hormone-independent. Approximately 13% (5 of 40) of the clones in the CSS group never arrested, suggesting *de novo* resistance. Supplementation with the synthetic androgen R1881 decreased the percentage of clones that

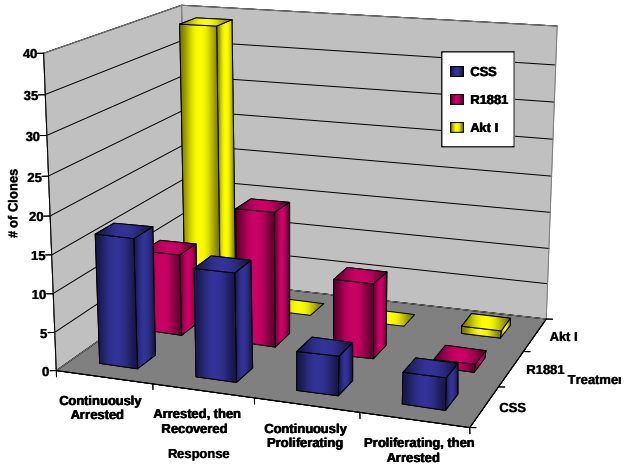

Fig. 1. LNCaP growth response to androgen-depletion. 40 LNCaP subclones were each grown long-term under conditions of androgen-depletion (CSS, blue), CSS media supplemented with the 1 nM of the synthetic, nonmetabolizable androgen R1881 (R1881, red), and CSS media supplemented with 10μM of the Akt inhibitor I (Akt I, yellow). Clones were assessed at weeks 5 and 10, and determined to be either arrested or proliferating at each point.

were continuously arrested compared to the CSS group, (only 28% compared to 43%), while increasing the number of clones that either recovered or continuously proliferated (18 (45%) and 10 (25%), respectively). In this current report, we present the results of our molecular analysis of these cells.

Evaluate cells for cell cycle, morphological, and molecular status

We have initiated studies investigating the molecular basis for results obtained with the hormone ablation study. By Western blot analysis, we evaluated the expression levels of a panel of proteins involved in cell cycle regulation and apoptosis, including cyclin D, p21 and p27. Surprisingly, we found no differences in the expression levels of these proteins (data not shown). Several studies have demonstrated an increase in expression of the androgen receptor (AR) at time of relapse, in both preclinical and patient samples. In agreement with these studies, we consistently observed an increase in AR expression levels in those clones that became hormone independent, compared to the levels observed in the controls (**Fig 2**, CS vs. Cont). Intriguingly, the clones exposed to the Akt inhibitor (Akt I) did not demonstrate this increase in AR levels, even though they were also grown in charcoal-stripped conditions. This was observed in all of the 14 hormone independent clones tested. We are currently conducting studies to determine whether suppression of AR expression is at the transcriptional or post-transcriptional level, and whether it is one of the key reasons that the Akt inhibitor-grown clones were unable to progress to hormone independence.

In addition to the changes in AR expression, we also observed that increased levels of the pro-survival protein, bcl-2, were not evident in the Akt I cells. As with the AR data, we are currently exploring the relevance of this observation in relationship to the efficacy of the Akt inhibitor at suppressing progression.

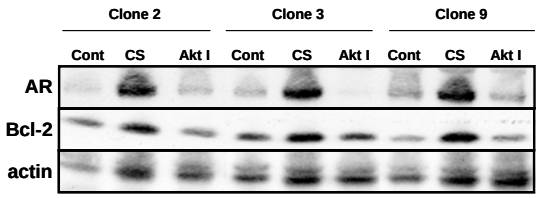

Fig. 2 Protein expression levels in selected LNCaP clones. Protein lysates from LNCaP clones for 10 weeks in complete serum (Cont), charcoal-stripped serum (CS) and CS with 10μM Akt inhibitor (Akt I) were analyzed by Western blot analysis for expression the androgen receptor (AR) and the pro-survival protein bcl-2 (Bcl-2). Actin was used as a loading control. Shown are examples of 14 clones examined.

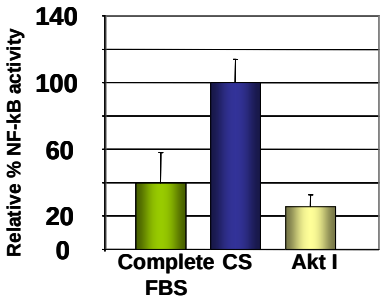

Fig. 3 NF-κB activity in LNCaP clones. LNCaP clones grown for 10 weeks in complete serum (Complete FBS, green bar), charcoal-stripped serum (CS, blue bar) and CS with 10μM Akt inhibitor (Akt I, yellow bar) were analyzed by luciferase assay for relative NF-κB activity using the 5X NF-κB luc reporter construct. All results were standardized to Renilla activity and are relative to results obtained in the CS clones. Shown is the combination of three independent experiments with 6 clones.

In addition to the AR, several reports have suggested that NF-κB signaling is also critical for prostate cancer progression to hormone independence. Because of this, we examined the hormone-independent clones for NF-κB activity (**Fig. 3**). We found that the clones that were hormone independent after long-term growth in androgen-depleted serum (CS, blue bar)) demonstrated almost 60% greater NF-κB activity compared to the same clones still hormone dependent grown in complete serum (Complete FBS, green bar). These same clones grown in the charcoal-stripped serum supplemented with 10μM of the Calbiochem Akt

inhibitor (Akt I, yellow bar) demonstrated significantly lower levels of NF-κB activity (30%) compared to either the complete or CS clones. These data suggest that increases in NF-κB activity may be critical for progression to hormone independence, and suppression of Akt activity may block this increase in activity. We addressed this question by determining whether suppression of NF-κB activity alters growth in hormone – independent cells.

To demonstrate the potential of targeting NF-κ B activity in suppressing hormone independence, BrDU incorporation assay was performed to measure cell proliferation and DNA synthesis in one of the hormone independent clone (LNCAP clone-2). As seen in **Figure 4**, cells were grown in either complete media (10% FBS), 10% charcoal-stripped FBS (CSS) or CSS with 10uM Parthenolide (an NF-kB inhibitor) for 10 weeks. Inhibition of NF-κB by parthenolide significantly suppressed cell proliferation in hormone-independent cells (**Fig 4A**). Protein levels in these cells were assessed after 10 weeks. As observed previously, AR and Bcl-2 expression were higher in CSS conditions as opposed to cells grown in FBS. However, parthenolide prevented this increased expression of these two proteins (**Fig. 4B**). In **Figure 4C**, cells demonstrated decreased nuclear localization of p65 (NF-κB subunit) with parthenolide treatment as compared to cells grown in FBS and CSS. Finally, apoptosis was assessed by activation of a pro-apoptosis marker, PARP, from the whole cell extract of these cells (**Fig. 4D**). Consistent with the proliferation assay, treatment with parthenolide increases apoptosis in hormone-depleted conditions. These results indicate that continuous exposure to parthenolide prevents cells from progressing to a hormone refractory state, correlating with a suppression of AR and Bcl-2 expression, suggesting that targeting NF-κB could be a potential therapeutic target in hormone-refractory prostate cancer.

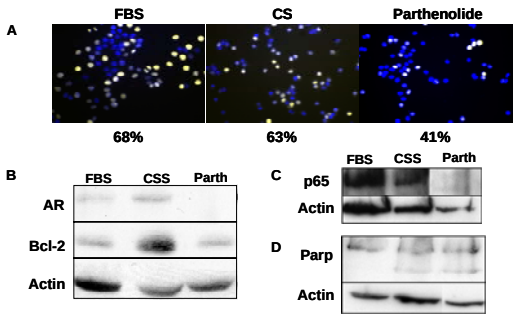

Fig. 4 Suppression of NF-kB Alters Hormone Independent Growth. Hormone-independent LNCaP clone 2 was grown for 10 weeks in Complete FBS (10% FBS), charcoal-stripped serum (CSS) and CSS with 10 uM parthenolide. *A.* Proliferation was assessed by BrDU incorporation (white cells are positive) *B.*, Expresion of AR and Bcl-2 were determined by Wetern blot analysis. *C.*, Expression of nuclear p65 was determined by Western blot analysis. *D.*, apoptosis was assessed by PARP cleavage (lower band is active form).

Since Akt inhibition effectively prevented progression to hormone independence, we were also interested in assessing its efficacy at inhibiting proliferation in hormone-independent cells. As seen in **Fig. 5**, we found that hormone-independent clones grown for 96 hours in either charcoal-stripped serum or

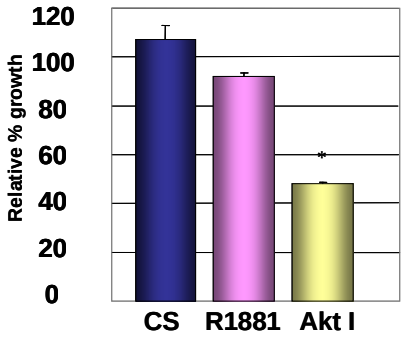

Fig. 5 Growth of hormone-independent LNCaP cells. Hormone-independent LNCaP clones grown for 10 weeks in charcoal-stripped serum were assessed by MTT analysis for proliferation when grown for 96 hours in charcoal-stripped serum alone (CS, blue bar), or CS supplemented with 1 μM of the synthetic androgen R1881 (R1881, pink bar), or 10 μM of the Calbiochem Akt inhibitor (Akt I, yellow bar). Shown is the combination of 3 independent experiments done with 6 clones. All results are relative to those obtained with the CS.

charcoal-stripped serum supplemented with 1 μM of the synthetic androgen R1881 demonstrated no significant difference in growth. Importantly, these same clones grown in the presence of 10 μM of the Akt inhibitor demonstrated an almost *60% decrease in proliferation*, as assessed by MTT analysis. These data strongly suggest that Akt activity is critical for continued proliferation/survival even once cells have proceeded to hormone independence, and that Akt remains a potential target for clinical intervention in the metastatic setting, even once the disease has relapsed.

In addition to these earlier studies, we have found that Akt regulates the activity of the enzymatic subunit of telomerase, hTERT, and that regulation of hTERT may play a role in mediating the effects of Akt in promoting progression. Activation of Akt activity in LNCaP and PC-3 cells increases hTERT mRNA levels (data not shown). Intriguingly, we have found that expression of higher levels of hTERT increases the clonogenic potential of both of these cell lines (**Fig. 7**). Soft agar assays were performed to determine the tumorigenic potential of infected LNCaP and PC-3 cells. hTERT overexpression in PC-3 and LNCaP cells significantly increased the colony forming ability of the cells by 50% in LNCaP cells and by 200% in PC-3 cells, suggesting that hTERT may play a role in promoting tumorigenicity of prostate cancer cells. We are currently pursuing studies to determine the contribution of hTERT to the tumorigenic properties of Akt.

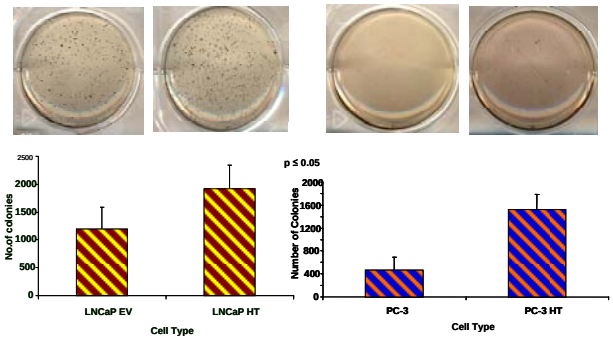

Fig. 7 hTERT Increases Tumorigenic Potential of LNCaP and PC-3Cells Tumorigenic potential of LNCaP cells was determined by growth in soft agar. Both cells lines were infected with either a control vector (EV) or an expression vector for hTERT (HT). Colony growth was counted after 1 week and is presented in bar format. Presented is the combination of three independent experiments.

Task 3*: To investigate in vivo whether Akt signaling is a critical component of the mechanism by which prostate cancer progresses to a condition of hormone independence.*

Initiate implantation of CWR22 tumor xenografts

Two years ago we reported that implantation of the CRW22 xenografts was to be initiated shortly. Unfortunately, as was reported last year, the original cells that were implanted into the mice were not hormone dependent. None of the tumors regressed upon removal of the testosterone pellet. We obtained new cells from the Gregory laboratory, and after extensive *in vitro* and *in vivo* testing for hormone dependence, we initiated the implantation and initial treatment regimen. In the next year, we will need to:

Complete treatment and tissue harvesting regimens
Molecular, immunohistochemical and cell cycle analyses of harvested tissues
Perform statistical analyses

KEY RESEARCH ACCOMPLISHMENTS:

- Development of several AR-positive hormone-independent prostate cancer LNCaP sublines
- Demonstration that inhibition of the Akt pathway results in suppression of expression and activity of key proteins involved in prostate cancer progression, including the androgen receptor, bcl-2 and NF-κB.
- Demonstration that Akt inhibition may be a realistic target for therapeutic intervention for the treatment of hormone-independent disease.

REPORTABLE OUTCOMES:

The data was presented at the annual meeting of the American Institute for Cancer Research as well as the annual meeting of the American Association for Cancer Research.

We have reported that:

1) Suppression of Akt activity precludes the ability of prostate cancer cell lines to progress to hormone independence.

2) Growth of hormone independent prostate cancer cells can be inhibited by suppression of Akt activity.

3) Akt suppression of progression is associated with inhibition of androgen receptor expression.

4) Suppression of progression is also associated with inhibition of NF-κB activity.

5) Inhibition of NF-κB activity suppresses hormone-independent growth, expression of AR, expression of Bcl-2 , and induces PARP activation (a marker of apoptosis).

6) Akt activity induces increased levels of hTERT mRNA, which may contribute to the more aggressive properties of Akt.

CONCLUSIONS:

As part of our on-going studies to better understand the role of the Akt kinase pathway in the progression of prostate cancer, we have found that treatment with an Akt inhibitor inhibited almost all progression to hormone independence in an *in vitro* model of androgen ablation. This was correlated with a suppression of expression and activity of key proteins involved in progression. These results suggest a critical role for Akt signaling in prostate cancer progression. The results of these *in vitro* studies will be confirmed using an animal model of prostate cancer progression in studies scheduled for the upcoming year.

The results of these studies could have a significant impact on clinical approaches for the treatment of recurrent prostate cancer. Currently, progression of prostate cancer to androgen independence remains the primary obstacle to improved survival with this disease. In order to improve overall survival, novel treatment strategies that are based upon specific molecular mechanisms that prolong the androgen-dependent state and that are useful for androgen-independent disease need to be identified. The results of our studies suggest that targeting the Akt pathway may provide such a strategy, resulting in increased survival among patients with recurrent disease.

U.S. Army Medical Research and Materiel Command Animal Use Report

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Contract Number: W81XWH-04-1-0218

This Report is for Fiscal Year 2006 (01 October 2005 - 30 September 2006)

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A. Animal	B. Number of animals purchased, bred, or housed but **not** yet used	C. Number of animals used involving no pain or distress	D. Number of animals used in which appropriate anesthetic, analgesic, or tranquilizing drugs were used to alleviate pain	E. Number of animals used in which pain or distress was not alleviated	F. Total Number of Animals (Columns C+D+E)
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Cats					
Guinea Pigs					
Hamsters					
Rabbits					
Non-human Primates					
Sheep					
Pigs					
Goats					
Horses					
Mice		60	0		60
Rats					
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Column A: List all animals used for the research, development, testing, evaluation, clinical investigations, diagnostic procedures, and/or instructional programs conducted. For the purpose of this report, an animal is defined as **any living nonhuman vertebrate**.

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Column C: Number of animals used in which the procedures did not cause more than slight or momentary pain or distress.

Column D: Number of animals used that were given analgesics, anesthetics, or tranquilizers to relieve pain or distress.

Column E: Number of animals used in painful procedures in which pain relieving compounds were not administered.

Column F: Sum of columns C, D, and E.

Forward this report by DECEMBER 01 of each year to:

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